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Induction of Apoptosis in HL-60 Human Promyelocytic Leukemia Cells by Adenosine A₃ Receptor Agonists

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Abstract

The effects of adenosine (ADO) analogs on cells of the human promyelocytic HL-60 line were examined. ADO A₃ receptor agonists, N⁶-(3-iodobenzyl) adenosine-5'-N-methylcarboxamide (IB-MECA, 30–60 μ M) and 2-chloro-N⁶-(3-iodobenzyl) adenosine-5'-N-methyluronamide (CI-IB-MECA, 10–30 μ M) induced apoptotic cell death. In contrast, neither an A₁/A₂ antagonist (XAC) nor other selective ADO receptor agonists (CPA, NECA and CGS21680) induced apoptosis at concentrations of ≥ 30 μ M. Both IB-MECA and CI-IB-MECA significantly induced Ca²⁺ release from intracellular Ca²⁺ pools followed by Ca²⁺ influx, suggesting the presence of phospholipase C-coupled ADO A₃ receptors on HL-60 cells. This was further supported by the presence of mRNA of ADO A₃ receptor in the cells. These results suggest that activation of ADO A₃ receptors is responsible for the ADO-induced apoptosis in HL-60 cells and could be of potential therapeutic value in the treatment of leukemia.

ADO regulates a variety of physiological effects on numerous cell types through activation of membrane-bound adenosine receptors. These have recently been classified into four distinct subtypes, A₁, A_{2a}, A_{2b} and A₃ (1). In particular, recent evidence indicates that these ADO receptors play roles in regulating development of cells by inducing apoptotic cell death (2–4). Apoptosis is an active self-destruction caused by activating a genetically programmed cascade of molecular events. This process leads to DNA degradation and death of the cell via nuclear and cytoplasmic breakup, packaging into apoptotic bodies, etc. (5, 6). In thymus, this process is essential for the development of T cells. ADO has recently been proposed to be involved in controlling the positive/negative selection of human thymocytes in relatively later stages of T cell development (2, 3). Thus, ADO induces apoptosis in mature CD3⁺CD4⁺CD8⁺ cells, as well as corticosteroid-sensitive CD4⁺CD8⁺ cells. These effects appear to be mediated by ADO receptors, since both A₁ and A₂ receptor agonists produced DNA fragmentation in these cells.

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In addition to the significant effects of ADO on thymocytes, recent report also showed that human promyelocytic HL-60 cells undergo apoptosis in response to treatment with ADO (4). HL-60 cells have been used extensively as an *in vitro* model for neutrophil function, because of their ability to differentiate into both functionally and morphologically mature myeloid cells (7, 8). Moreover, because a variety of chemicals, radiation and hyperthermia induce apoptosis (4, 9, 10), this model is also useful to study mechanisms of apoptosis of differentiating cells in myelocytic lineage. Tanaka et al. (4) indicated that ADO but not other naturally occurring nucleosides induced apoptosis in HL-60 cells. Curiously, selective ADO receptor agonists failed to induce apoptosis in these cells, thus the authors concluded that ADO-induced apoptosis in HL-60 cells is not likely mediated by ADO-receptors. However, these authors did not take into account the novel A₃ adenosine receptor, since selective agents for this subtype were not available until recently (11). The present study indicates that selective ADO A₃ receptor agonists, IB-MECA (11) and CI-IB-MECA (12) synthesized in our laboratory, induced receptor-mediated Ca²⁺ influx as well as apoptotic cell death in HL-60 cells.

MATERIALS AND METHODS

Reagents

RPM 1640 medium and fetal bovine serum were obtained from Gibco BRL (Gaithersburg, MD). Erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA). ADO, XAC, CPA, NECA and CGS21680 were purchased from RBI (Natick, MA). Highly selective ADO A₃ agonists, IB-MECA (11) and CI-IB-MECA (12), were synthesized in our laboratory. Chromomycin A3 was purchased from Sigma Chemical (St. Louis, MO).

Cell cultures and preparations

The HL-60 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum. 100 units/ml penicillin. 100 mg/ml streptomycin and 2 mM L-glutamine. The cells were split every third day, and 2 days before each experiment cultures were diluted to 2×10^5 cells/ml. For analysis of DNA content, aliquots of 2 ml were placed into 12-well flat-bottomed plates (Costar, Cambridge, MA, USA) containing 2 ~ 6 μ l test-compound solutions at defined concentrations or 6 μ l DMSO (diluting medium).

DNA content analysis by flow cytometry

Cells were fixed by adding $\sim 10^7$ cells suspended in 1 ml of PBS to 1 ml 80% ethanol at -20°C and stored for 48–120 hrs. After the cells were washed twice with PBS, the cells were stained with 20 mg/ml chromomycin A3 dissolved in PBS containing 2 mM MgCl₂ by incubation in subdued light (30 min; 4°C). The cells were then analyzed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) as previously described (13).

DNA gel electrophoresis

DNA fragmentation was analyzed as previously described (14). In brief, cells were washed twice with PBS at 4°C , resuspended in lysis buffer (5 mM Tris-HCl, pH 8.0, 20 mM EDTA tetrasodium salt tetrahydrate and 0.5% Triton X) and homogenized with a disposable Dounce homogenizer (Kontes, Vineland, NJ). Homogenates were incubated for 2 hours at

4°C and centrifuged at 15000g (4°C). The supernatants were collected and treated with 100 µg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN) for 1 hour at 56°C. Following phenol/chloroform extraction and ethanol precipitation, the DNA samples were resuspended in deionized water, treated with Rnase (100 µg/ml), and electrophoresed on 1% agarose gel. A molecular weight standard run in parallel consisted of ϕ X174 RF DNA/Hae III fragments (Life Technologies, Gaithersburg, MD).

RT-PCR analysis

Total RNA was prepared by the single-step method (15) with slight modifications (RNA Stat-60, Tel-Test “B”, Inc., Friendswood, TX). One µg of total RNA was incubated at room temperature for 15 min with 1 unit of RNase-free DNase 1 (Boehringer Mannheim, Indianapolis, IN), and the first strand cDNA was synthesized with 0.5 µg of oligo (dT)_{12–18} primer and 200 units of the cloned M-MLV reverse transcriptase (SUPERScript II preamplification system, Life Technologies, Gaithersburg, MD).

The human A₃ ADO receptor sequence was amplified with 5′ primer sequence (ACCCCATGTTTGGCTG) and 3′ primer sequence (GCACAAGCTGTGGTACCTCA) giving a 361 bp product (16, 17). PCR was carried out in 50 µl and at the following conditions: the initial denaturing step at 95°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30s, 72°C for 30 s, except the last elongation at 72°C for 10 min. Subsequently, 10 µl of PCR products were run on the 4% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME) and examined by ethidium bromide staining.

Measurement of cytosolic Ca²⁺ concentration

HL-60 cells (10⁶ cells/ml) were incubated in the presence of 1µg fluo-3AM in a 1:1 mixture of RPMI 1640 and Hanks’ balanced salt solution (HBSS) buffer for 40 min at room temperature.

The cells were suspended in a 1:1 mixture of RPMI medium and HBSS buffer at about 10⁶ cells/ml. Ten ml of a DMSO solution containing 1 µg/ml of fluo-3 AM (Molecular Probes, Inc., Eugene OR) was added to 10 ml of the cell suspension, and cells were left in the dark for 40 min at room temperature. The cells were centrifuged at 1200 rpm for 10 min to remove extracellular dye and resuspended in HBSS. Two ml of cell suspension was added to each cuvette with a magnetic stir bar, and the fluorescence intensity of fluo-3 AM was quantified using a Deltascan fluorescence spectrophotometer (Photon Technology International, Inc., Brunswick, NJ) with the excitation wavelength set at 506 nm and emission wavelength monitored at 526 nm. The maximum fluo-3 fluorescence (F max) in HL-60 cells was determined by adding Triton-X (0.025%) or 4-bromoA23187 (1 µM) in the presence of 1 mM Ca²⁺. The minimum fluorescence (F min) was determined following quenching of fluo-3 with 0.5 mM EDTA.

RESULTS AND DISCUSSION

Fig. 1 shows the changes in DNA frequency distribution histograms of HL-60 cells exposed to the A₃ receptor agonists, IB-MECA (10, 30, or 60 µM) and CI-IB-MECA (10 or 30 µM), for 48 hrs. The DNA frequency histogram shows that the treatment with IB-MECA or CI-

IB-MECA at 10 or 30 μM produced cells with hypodiploid DNA content. XAC (50 μM) did not inhibit the DNA fragmentation induced by CI-IB-MECA (data not shown). This DNA fragmentation is typical of apoptosis, thus the A_3 receptor agonists induced apoptosis in these cells. This finding is consistent to the results of DNA gel electrophoresis (Fig. 2) which show that HL-60 cells revealed internucleosomal DNA fragmentation when cells were cultured with 30 μM CI-IB-MECA or 30 μM CADO for 48 hr. The same results were observed when the cells were incubated for 48 hr with 150 nM camptothecin as an active control (Fig. 2), as was shown previously in HL-60 cells (18). ADO alone at a concentration of 100 μM did not induce apoptosis (data not shown), but in the presence of 100 μM EHNA, an ADO deaminase inhibitor, apoptosis occurred (Fig. 1B).

The ADO receptor agonists, CPA (A_1 -selective), NECA (non-selective), and CGS21680 (A_{2a} -selective), and an ADO A_1/A_2 antagonist, XAC, did not induce apoptosis at concentrations of 10–50 μM (data not shown).

CADO (an A_1 agonist of low selectivity) also elicited apoptosis at 10 μM (Fig. 1E). However, XAC (50 μM) had no effect on frequency of cells with fractional DNA content induced by CADO (data not shown).

The RT-PCR analysis clearly indicated the expression of ADO A_3 receptor mRNA in the HL-60 cells. Analysis of cDNA, using primers designed to specifically amplify a fragment of the human A_3 receptor cDNA, revealed the expected 361-bp PCR product (Fig. 3). No signal for the human A_3 receptor was detected in the control without reverse-transcriptase (Lane 3), or in another lane (4) in which water was substituted for the cDNA (Fig. 3).

A_3 receptor agonists, IB-MECA and CI-IB-MECA (10 and 30 μM), produced increases in concentrations of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) (Fig. 4A). Both drugs produced a rapid rise followed by a sustained increase in $[\text{Ca}^{2+}]_i$. Chelation of extracellular Ca^{2+} with EGTA (0.5 mM) did not affect the initial rise in $[\text{Ca}^{2+}]_i$ but attenuated a second phase of elevations in $[\text{Ca}^{2+}]_i$. Thus, the first rapid increase in $[\text{Ca}^{2+}]_i$ was due to Ca^{2+} release from intracellular Ca^{2+} pools, while the second phase appeared to result from an influx of extracellular Ca^{2+} (Fig. 4A). Although NECA (30 μM) and CADO (30 μM) tended to induce an increase in intracellular Ca^{2+} levels, the intensity of the elevation of $[\text{Ca}^{2+}]_i$ was clearly lower than that of CI-IB-MECA (30 μM). At concentrations of 30 μM , CPA, CGS21680 and XAC had no effect on intracellular Ca^{2+} levels (data not shown).

In the present study we have examined the effect of ADO and a variety of its analogs on HL-60 cells. ADO (100 μM) in the presence of an ADO deaminase inhibitor, EHNA (100 μM), caused apoptosis (Fig. 1). However, ADO receptor agonists CPA (A_1 -selective) and CGS21680 (A_2 -selective) did not have any detectable effects on the cells. Also, the non-selective A_1 , A_{2a} agonist NECA did not induce apoptosis. Therefore, the A_1 , A_{2a} or A_{2b} ADO receptor(s) alone do not seem to be involved in the cell death. These findings are consistent with the results obtained by Tanaka et al (4). However, in the concentration range of 10–30 μM , selective ADO A_3 receptor agonists, IB-MECA and CI-IB-MECA, apparently induced apoptosis. CI-IB-MECA was more potent in inducing apoptosis than IB-MECA. The concentrations required for eliciting apoptosis in HL-60 cells are considerably higher

than the IC_{50} values ($\sim 10^{-7}$ M) for rat A_3 -receptor mediated adenylyl cyclase (12), possibly reflecting differences between whole cells and membranes or between species. The affinity of NECA, which did not induce apoptosis, at A_3 receptors is >100-fold lower than IB-MECA (24). Two lines of evidence suggest the presence of functional ADO A_3 receptors on HL-60 cells. First, both IB-MECA and CI-IB-MECA produced Ca^{2+} release from the intracellular Ca^{2+} pools followed by a sustained Ca^{2+} influx, which is typical of phospholipase C-coupled receptor-mediated Ca^{2+} response. A phospholipase C-coupled ADO A_3 receptor-mediated Ca^{2+} response has also been demonstrated in rat basophilic cells (RBL-2H3) (19), and A_3 agonists were found to activate phospholipase C in rat brain slices (29). Second, the mRNA of ADO A_3 receptor is apparently expressed in the HL-60 cells. Therefore, overall results suggest that activation of ADO A_3 receptors is responsible for ADO-induced apoptosis in HL-60 cells.

In apparent contradiction to the above hypothesis, CADO, an A_1 agonist of low selectivity, was able to induce apoptosis in HL-60 cells, as it was recently reported to do also in thymocytes (3) and in astroglial cells in rat brain primary cultures (20). Since CADO (26, 27) is equipotent to NECA at A_1 receptors (9.3 vs. 6.3 nM) and less potent than NECA at A_{2a} (63 vs. 10 nM), A_{2b} (24 vs. 2.6 μ M), and A_3 (1890 vs. 113 nM) receptors, it is likely that the mechanism of apoptosis induced by CADO is not via adenosine receptors. Tanaka et al. (4) observed an effect of ADO but not CADO on polyADP ribosylation activity in HL-60 cells, further supporting distinct mechanisms for cell death induced by these two analogues. The hypothesis of a non- A_1/A_2 receptor-mediated pathway for the action of CADO is further supported by our finding that an ADO A_1/A_2 antagonist XAC had no effect on CADO-induced apoptosis in HL-60 cells. It is also noteworthy that a closely related compound, 2-chloro-2'-deoxyadenosine (cladribine), also induces apoptosis in leukemic cells (28), probably in an ADO receptor-independent fashion since it binds only very weakly to the receptors (26). Cladribine is currently being used in the treatment of leukemia (21). Detailed mechanisms responsible for the induction of apoptosis by either CADO or A_3 agonists are now under investigation.

Recent studies have identified a novel adenosine receptor subtype, A_3 , in the rat (22) and similar receptors have been cloned from both human (17) and sheep brain cDNA libraries (23). Few physiological functions of ADO A_3 receptor are known (24). In particular with respect to immunologic function, there is little information available except the reports cited that activation of A_3 receptors regulates function in basophilic cells (19) and mast cells (25). Further elucidation of the mechanisms and control of apoptosis in hematopoietic cells is likely to shed new light on the pathophysiology of inflammation and suggest new approaches to the therapy of inflammatory diseases and leukemia.

Abbreviations used

ADO	adenosine
CADO	2-chloroadenosine

CGS 21680	2-[4-[(2-carboxyethyl)-phenyl]ethylamino]-5' N-ethylcarboxamidoadenosine
CI-IB-MECA	N ⁶ -(3-iodobenzyl)-2-chloro-adenosine-5'-N-methyluronamide
CPA	N ⁶ -cyclopentyladenosine
IB-MECA	N ⁶ -(3-iodobenzyl)adenosine-5'-N-methyluronamide
NECA	5'-N-ethylcarboxamidoadenosine
Tris	tris (hydroxymethyl)aminomethane
XAC	(8-[4-[[[(2-aminoethyl)amino]-carbonyl]methyl]oxy]phenyl)-1,3-dipropylxanthine)

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FIG. 1.

Flow cytometric DNA analysis of HL-60 cells determined by fluorescent cell sorting. The histograms were generated using chromomycin A3-stained cells that had been pretreated for 48 h with various adenosine agonists. Control cells are shown in A, and agonists used were (B) 100 μ M ADO (in the presence of the adenosine deaminase inhibitor EHNA, 100 μ M), (C) IB-MECA (10–60 μ M, as shown), (D) CI-IB-MECA (10 or 30 μ M, as shown), (E) CADO (10 or 30 μ M, as shown). The horizontal and vertical axes refer to log fluorescence intensity and relative cell number, respectively.

A B C D

FIG. 2.

Agarose gel electrophoresis of DNA from cells treated with (Lane A) CADO (30 μ M), (Lane B) CI-IB-MECA (30 μ M), (Lane C) camptothecin (150 nM), or (Lane D) Control (strong spot in lower MW range is likely partially degraded RNA).

FIG. 3.

RT-PCR products obtained from HL-60 cells and separated on a 4% NuSieve 3:1 agarose gel. The PCR product in Lane 2 is 361 bp long (situated between molecular weight markers for 310 and 613 bp), according to the human A₃ adenosine receptor sequence. (Lane 3) Control without reverse-transcriptase, and (Lane 4) water substituted for the cDNA.

FIG. 4.

Effect of adenosine agonists on intracellular Ca^{2+} levels in HL-60 cells. Cells were prelabeled with Fluo-3AM, as described in Materials and Methods. Effects of CI-IB-MECA (30 μ M) in the absence or presence of 0.5 mM EGTA. The basal $[Ca^{2+}]$ level was calculated to be in the range of 40–200 nM.